**PCT**

| (51) International Patent Classification 5 :<br>C12N 15/11, A61K 31/70<br>C12N 9/00, C12Q 1/68<br>// A61K 48/00 | A2 | (11) International Publication Number: **WO 93/22434**<br><br>(43) International Publication Date: 11 November 1993 (11.11.93) |
|---|---|---|

(54) Title: TARGETED CLEAVAGE OF RNA USING EUKARYOTIC RIBONUCLEASE P AND EXTERNAL GUIDE SEQUENCE

(57) Abstract

It has been discovered that any RNA can be targeted for cleavage by RNAase P from eukaryotic cells, for example, human HeLa cells, using a suitably designed oligoribonucleotide ("external guide sequence", or EGS) to form a hybrid with the target RNA, thereby creating a substrate for cleavage by RNAase P *in vitro*. There are two classes of EGS that can target RNA for cleavage by human RNAase P. These are prepared by transcription from a small DNA fragment that contains a sequence coding for an RNA that can assume a tRNA-like secondary structure. In the first class, the structure lacks at least the first thirteen nucleotides from the 5' terminus of the tRNA-like sequence, the anticodon stem and loop, the variable loop or part of the variable loop. The most efficient EGS with human RNAase P is the EGS in which the anticodon stem and loop was deleted. In the second class, the EGS has changes in both the equivalent of the T-loop and the anticodon stem of the tRNA-like segment of the EGS. Methods are also disclosed to randomly select and to express a suitable EGS *in vivo* to make a selected RNA a target for cleavage by the host cell RNAase P, thus preventing expression of the function of the target RNA. The methods and compositions should be useful to prevent the expression of disease-causing genes *in vivo*.

## TARGETED CLEAVAGE OF RNA USING EUKARYOTIC
## RIBONUCLEASE P AND EXTERNAL GUIDE SEQUENCE

### Background of the Invention

This invention is in the general area of genetic
engineering of nucleic acid sequences, especially RNA
sequences that are substrates for Ribonuclease P.

5      This is a continuation-in-part of U.S. Serial
No. 07/931,937 filed August 18, 1992 entitled
"Targeted Cleavage of RNA Using Eukaryotic
Ribonuclease P and External Guide Sequence", by Yan
Yuan, Cecilia Guerrier-Takada and Sidney Altman, which
is a continuation in part of 07/875,099 filed April

10     28, 1992; which is a continuation in part of U.S.
Serial No. 07/568,834 entitled "Therapeutic Ribozyme
Compositions" filed August 1990 by Sidney Altman, et
al., issued as U.S. Patent No. 5,168,053, which is a
continuation in part of U.S. Serial No. 07/328,368

15     entitled "Removing or Inactivating Undesired RNA"
filed March 24, 1989 by Sidney Altman, Anthony C.
Forster, and Cecilia L. Guerrier-Takada, now
abandoned.

The United States government may have certain
20     rights in this invention as a result of grants from
the National Institutes of Health and National Science
Foundation, GM 19422 and DMB 9101670 to Sidney Altman.

Discoveries in the realm of molecular biology
over the past five years have led to the realization
25     that RNA has a series of distinct capabilities and
biological activities previously unsuspected.  The
most important of these novel RNA-level discoveries
has been the finding that RNA can be an enzyme as well
as an information carrier.

30     There are several classes of ribozymes now known
which are involved in the cleavage and/or ligation of
RNA chains.  A ribozyme is defined as an enzyme which
is made of RNA, most of which work on RNA substrates.
Ribozymes have been known since 1982, when Cech and

colleagues (Cell, 31: 147-157) showed that a ribosomal
RNA precursor in *Tetrahymena*, a unicellular eukaryote,
undergoes cleavage catalyzed by elements in the RNA
sequence to be removed during the conversion of the
rRNA precursor into mature rRNA. This sequence to be
removed (called an intervening sequence or intron) is
one of what are now known to be numerous examples of
"Class I" intron ribozyme activities. A similar
"Class II" intron ribozyme mechanism was discovered
more recently, involving the cleavage and subsequent
ligation of a number of yeast mitochondrial RNAs
(Nature, 324: 429-433 1987). Cech and colleagues
described certain *in vitro* applications of "class I"
ribozymes in PCT/US887/03161 by University Patents,
Inc., (published as WO 88/04300 16 June 1988). Their
potential for therapeutic applications in cells and in
patients remains unclear.

A third class of ribozyme, discovered in 1983,
was the first to be shown to work in trans (i.e., to
work under conditions where the ribozyme is built into
one RNA chain while the substrate to be cleaved is a
second, separate RNA chain). This ribozyme, called M1
RNA, was characterized in 1983 by Altman and
colleagues as responsible for the cleavage which forms
mature 5' ends of all transfer RNAs (tRNAs) in E.
coli. Analogous RNA-containing enzymes concerned with
tRNA synthesis have since been found in all cells in
which they have been sought, including a number of
human cell lines, though the relevant eucaryotic RNAs
have not yet been shown to be catalytic by themselves
*in vitro*.

The discovery and characterization of this
catalytic RNA is reviewed by Sidney Altman, in
"Ribonuclease P: An Enzyme with a Catalytic RNA
Subunit" in Adv. Enzymol. 62, 1-36 (1989). The
activity was first isolated from *E. coli* extracts, and
subsequently determined to be a ribonucleoprotein

having two components, an RNA component called M1 and a protein component called C5. The RNA cleaved substrates in a true enzymatic reaction, as measured using Michaelis-Menton kinetics. M1 was determined to be solely responsible for substrate recognition and C5 was determined to alter $k_{cat}$ but not $K_M$, as reported by Guerrier-Takada, et al., Cell 35, 849 (1983) and McClain, et al., Science 238, 527 (1987). Sequencing showed that M1 RNA is 377 nucleotides long, $M_r$ approximately 125,000, and that the protein consists of 119 amino acids, $M_r$ approximately 13,800, as reported by Hansen, et al., Gene 38, 535 (1987).

Two other ribozyme classes are related to the replication cycle of a group of self-replicating RNAs called "viroid-like pathogens", or VLPs. Plant viroids, RNA satellites of plant viruses, and Hepatitis delta virus are all members of the VLP group. The VLPs can be divided into two classes: Class I, free living viroids; and Class II, including virusoids and satellite viroids (RNA molecules which require a helper virus to replicate). The hepatitis delta virus is a Class II VLP by this definition.

In 1984, Branch and Robertson (Science, 233: 450-455) published the replication cycle strategies for these pathogens, subsequently verified by experiments conducted in several laboratories. A key element of this "rolling-circle" replication strategy is that the VLP undergoing replication makes greater-than-unit-length copies of its information, which are then cleaved to monomeric size by ribozyme activities built into the RNA of the VLP itself. Sharmeen at. al., J. Virol., 62, 2674-2679 (1988); Branch, et. al., Science, 243, 649-652 (1989); and Wu and Lai, Science 243, 652-655 (1989), defined the ribozyme cleavage points of both delta strands and the domains containing them for hepatitis delta virus.

4

One type of VLP ribozymes is defined by a small
structural domain, consisting of only about 30
nucleotides, called a "hammerhead". Uhlenbeck, _Nature_
(1987), first developed these small (as few as 18
5   nucleotides) and relatively specific ribozyme
sequences from plant viroids such as avocado sunblotch
viroid and the satellite RNAs of tobacco ringspot
virus and lucerne transient streak virus. Uhlenbeck
(1987) and Forster and Symons (_Cell_ 50, 9-16, 1987),
10  defined the requirements for cleavage by this ribozyme
class. Various embodiments and potential applications
have also been described by Haseloff, Gerlach and
Jennings in PCT/AU88/00478 by Commonwealth Scientific
and Industrial Research Organization (published as WO
15  90/05852 29 June 1989).

All reactions that are governed by RNA _in vivo_
result in the transesterification or hydrolysis of
specific phosphodiester bonds in RNA. In several
classes of these reactions, an intramolecular site of
20  cleavage or ligation is identified by internal guide
sequences (IGSs) which form base pairs with the
segment of the phosphodiester chain that contains the
cleavage site. The _Tetrahymena_ sequence, as well as
the subsequently discovered sequence in yeast, is not
25  a true enzyme since it is not regenerated in the
process but instead acts in a stoichiometric ratio.
Although it is possible to engineer fragments of this
sequence which have enzymatic activity under certain
conditions _in vitro_ and are able to cleave and ligate
30  RNA, a disadvantage to these fragments is that they
are very large (requiring more than 200 residues of
the original 415 nucleotide sequence) and of limited
specificity. In their present forms, the _Tetrahymena_
ribozymes have four-base recognition sequences and the
35  hammerhead ribozymes have approximately 12-base
recognition sequences, allowing these ribozymes to be
used in a complementary fashion to cleave RNA.

IGSs are not present in one class of reactions
governed by RNA that is enzymatic *in vivo*, cleavage of
precursor tRNA molecules by the RNA component of
eubacterial RNAase P, described by Guerrier-Takada, et
al., _Cell_ 35, 849 (1983) and reviewed by Altman, <u>Adv.
Enzymol.</u> 62, 1 (1989).  The nucleotide sequence of the
segment of the phosphodiester chain that contains the
cleavage site is not conserved among different
substrates for RNAase P, so it cannot be recognized as
a unique IGS for the enzyme.

U.S. Serial No. 07/568,834 entitled "Therapeutic
Ribozyme Compositions" filed August 1990 by Sidney
Altman, et al., and U.S. Serial No. 07/328,368
entitled "Removing or Inactivating Undesired RNA"
filed March 24, 1989 by Sidney Altman, et al.,
disclose that it is possible to target any RNA
molecule for cleavage by bacterial RNAase P by forming
a nucleotide sequence part of which is complementary
to a targeted site and which includes a terminal 3'-
NCCA, wherein the sequence is designed to hybridize to
the targeted RNA so that the bacterial RNAase P
cleaves the substrate at the hybrid base-paired
region.  Specificity is determined by the
complementary sequence.  The sequence is preferably
ten to fifteen nucleotides in length and may contain
non-complementary nucleotides to the extent this does
not interfere with formation of several base pairs by
the complementary sequence which is followed by NCCA
at the 3' end.

Subsequent studies have demonstrated that the
external guide sequence, or "EGS", that is useful in
targeting procaryotic RNAase P, does not result in
cleavage of a targeted RNA strand by eukaryotic RNAase
P.

It is therefore an object of the present
invention to provide methods and compositions for
specifically cleaving targeted RNA sequences using

eukaryotic RNAase P or functional equivalents thereof.

It is a further object of the present invention
to provide methods and compositions for specifically
cleaving RNA, both *in vitro* and *in vivo* within
eukaryotic cells, for the treatment of disease
conditions which involve RNA transcription or
translation, such as diseases caused by RNA and DNA
viruses and expression of excessive or pathogenic
proteins from mRNA, or of excessive or pathogenic RNA,
itself.

## Summary of the Invention

It has been discovered that any RNA can be
targeted for cleavage by RNAase P from human cells,
using a suitably designed oligoribonucleotide
("external guide sequence", or EGS) to form a hybrid
with the target RNA, thereby creating a substrate for
cleavage by RNAase P *in vitro*. The data indicate that
it is reasonable to conclude that these observations
can be extended to RNAase P from any eucaryotic cell.
There are two classes of EGS that can target RNA for
cleavage by human RNAase P. These are prepared by
transcription from a small DNA fragment that contains
a sequence coding for an RNA that can assume a tRNA-
like secondary structure. In the first class, the
structure lacks at least the first thirteen
nucleotides from the 5' terminus of the tRNA-like
sequence, the anticodon stem and loop, or the variable
loop or part of the variable loop. The most efficient
EGS of this first class with human RNAase P is the EGS
in which the anticodon stem and loop was deleted. In
the second class, the EGS has changes in the
equivalent of the T-loop, the variable loop, and the
anticodon stem of the tRNA-like segment of the EGS.
Methods are also disclosed to select a suitable EGS of

the second class for use *in vivo* to make a selected
RNA a target for cleavage by the host cell RNAase P,
thus preventing expression of the function of the
target RNA.   The methods and compositions should be
useful to prevent the expression of disease-causing
genes *in vivo*.

## Brief Description of the Drawings

Figure 1 is the sequence and structure of H1
RNA.

Figure 2 is a general scheme of an EGS for use
in targeting eukaryotic RNAase P to cleave a specific
substrate, with the arms and stems characteristic of a
tRNA labeled.

Figure 3a is the sequence and secondary
structures of the precursor to tRNA$^{Tyr}$ and a complex of
a substrate with an EGS derived tRNA$^{Tyr}$.  pTyr: *E. coli*
tRNA$^{Tyr}$ precursor; pAva: substrate (target) RNA with 5'
leader sequence and the first fourteen nucleotides of
*E. coli* tRNA$^{Tyr}$.  Figure 3b is an autoradiograph of a
gel electrophoresis analysis of the cleavage reaction.
Arrows mark the expected cleavage sites.

Figure 4 is a schematic representation of
complexes of chloramphenicol acetyltransferase mRNA
and the EGS$^{CAT}$ RNAs.

Figures 5a and 5b are schematics of one class of
EGS for use in targeting eukaryotic RNAase P to cleave
a specific substrate:  Figure 5a is an EGS
corrresponding to the EGS in Figure 2; Figure 5b is an
EGS where the anticodon stem and loop have been removed.

Figures 6a, 6b, and 6c are schematics of a
second class of EGS for use in targeting eukaryotic
RNAase P to cleave a specific substrate where the
extra loop, the T loop, and the anticodon stem have
been modified.

8

## Detailed Description of the Invention

Ribonuclease P from *E. coli* can cleave
oligoribonucleotides that are found in hydrogen-bonded
complexes that resemble the aminoacyl stem and 5'
leader sequence of tRNA precursors. RNAase P from
human (HeLa) cells cannot cleave *in vitro* the 5'
proximal oligoribonucleotide in such simple complexes
but can do so when the 3' proximal oligoribonucleotide
(external guide sequence or EGS) resembles portions of
a tRNA molecule.

Examples provided below show the efficiency of
cleavage of the mRNA for chloramphenicol
acetyltransferase (CAT) by human RNAase P. In such a
complex, the sequence of the 5' oligoribonucleotide,
as well as that of the EGS, depend on the choice of
target site in the mRNA. The presence of the
appropriately designed EGS efficiently reduces CAT
enzymatic activity *in vivo*, as well as promotes
cleavage of CAT mRNA *in vitro*, indicating that this
method should be of general use for gene inactivation.

### Ribonuclease P.

Ribonuclease P is an enzyme consisting of
protein and RNA subunits that cleaves tRNA precursors
to generate the 5' termini of tRNAs. This essential
enzymatic activity has been found in all cell types
examined, both prokaryotic and eukaryotic. During the
studies on recognition of the substrate by RNAase P,
it was found that *E. coli* RNAase P can cleave
synthetic tRNA-related substrates that lack specific
domains (the D, T and anticodon stems and loops) of
the normal tRNA structure. A half-turn of an RNA
helix and a 3' proximal CCA sequence contain
sufficient recognition elements to allow the reaction
to proceed. The 5' proximal sequence of the RNA helix
does not have to be covalently linked to 3' proximal
sequence of the helix. The 3' proximal sequence of

the stem can be regarded as an "external guide
sequence" (EGS) because it identifies the site of
cleavage in the 5' proximal region through a base-
paired region.

5          Theoretically, any RNA of known sequence could
be targeted by a custom-designed EGS RNA for specific
cleavage by RNAase P.  EGS-directed mRNA cleavage has
been achieved with *E. coli* RNAase P *in vitro*.

          RNAase P from *E. coli* and human cells have
10     similar but not identical biochemical properties.
Their RNA components have similar secondary
structures.  However, the substrate range of human
RNAase P is much narrower than that of the *E. coli*
enzyme.  For example, although *E. coli* RNAase P can
15     cleave a synthetic tRNA-related substrate that lacks
three specific domains (the D, variable, and anticodon
stem and loop) of the normal tRNA structure, it is not
a substrate for the human enzyme or that from the
yeast, *S. cerevisiae*.  Therefore, the simplest design
20     of an EGS for RNA cleavage by RNAase P from human and
presumably other eukaryotic cells has to be different
from that for cleavage by *E. coli* RNAase P.

          As used herein, unless otherwise specified,
RNAase P refers to the RNAase P in the cell in which
25     the RNA to be cleaved is located, whether endogenous,
added to the cell, or as used *in vitro*. Many of the
techniques described herein are known to those skilled
in the art, as are methods for making, and sources of,
reagents.  The teachings of any references cited
30     herein with respect to methods and reagents are
specifically incorporated herein, as well as for the
purpose of demonstrating the scope and level of skill
in the art.

**The External Guide Sequence**
35     **A.   RNAase P targeting Sequence.**

          As shown by Figures 2, 3, 4, 5 and 6, an EGS for
a human RNAase P consists of a sequence which, when in

10

a complex with the target substrate molecule, forms a
secondary structure resembling that of a tRNA
cloverleaf or a substantial part of it.  As used
herein, the term "resembling a precursor tRNA" means a
5    sufficient portion of the tRNA secondary structure to
form a complex which will result in cleavage of the
target RNA by RNAase P.  The sequence of the EGS can
be derived from any tRNA except that the D stem and
aminoacyl stem have to be altered to be complementary
10   to the target substrate sequence and the apparent D
stem contains four base pairs.  The presence of the
3'-CCA enhances the efficiency of the reaction with
the human RNAase P by about 35%.  The anticodon loop
and stem and extra loop can separately be deleted and
15   the T loop and stem can be modified without decreasing
the usefulness of the EGS and, in the case of the
anticodon stem and loop deletion, increases the
efficiency of the reaction by about ten fold.  Changes
in other parts of an EGS (see Figure 6) can increase
20   its efficiency about one hundred fold.

        The desired secondary structure is determined
using conventional Watson-Crick base pairing schemes
to form a structure resembling a tRNA, i.e., having
structure as described below.  The specific sequence
25   of the hydrogen bonded regions is not so critical, as
long as the desired structure is formed.  All tRNAs,
including tRNAs from a wide variety of bacteria and
eukaryotes, conform to the same general secondary
structure.  This is typically written in the form of a
30   cloverleaf, maintained by hydrogen-bonded base pairing
between short complementary regions.  The four major
arms are named for their structure or function:  The
acceptor arm consists of a 3' terminal $CCA_{OH}$ plus a
variable fourth nucleotide extending beyond the stem
35   formed by base-pairing the 5' and 3' segments of the
molecule.  The other arms consist of base-paired stems
and unpaired loops.  The "T" arm is named for the

11

presence of the ribothymidine nucleotide and contains
seven unpaired bases.  The anticodon arm always
contains the anticodon triplet in the center of the
loop and consists of seven unpaired bases.  The D arm
is named for the presence of the base dihydrouridine,
another of the modified bases in tRNA, and includes
between eight and twelve unpaired bases.  Positions
are numbered from 5' to 3' according to the most
common tRNA structure, which has 76 residues.  The
overall range of tRNA lengths is from 74 to 95 bases.
The variation in length is caused by differences in
the structure of two of the arms, the D arm and the
extra arm, which lies between the T and anticodon
arms, which can contain between three and five bases,
or between 13 and 21 bases with a stem of about five
bases.  The base pairing that maintains the secondary
structure is virtually invariant:  there are always
seven base pairs in the acceptor stem, five in the T
arm, five in the anticodon arm, and three or four in
the D arm.

As used herein, a hybrid structure having
secondary structure resembling a precursor tRNA under
conditions promoting cleavage by the RNAase P of the
substrate at the nucleotide at the 5' end of the base-
paired region, preferably includes a D loop and stem,
an aminoacyl stem, an anticodon loop and stem, a
variable loop and stem, and a T loop and stem, where
the latter three may be modified compared to the
sequence and detailed structure found in the parent molecule.

A few nucleotides are always found in the same
positions in 90 to 95% of tRNAs, with some additional
nucleotides being semiconserved (or semivariant).
This is not a requirement in the EGS, as long as the
sequence is complementary to the target and forms the
secondary structure characteristic of the tRNA.  As
shown in Figure 2, the sequence forming the aminoacyl

12

stem and D loop and stem are changed in the EGS to be
complementary to the target RNA.

The base paired double-helical stems of the
secondary structure are maintained in the tertiary
structure, creating two double helices at right angles
to each other. The acceptor stem and the T stem form
one continuous double helix with a single gap; the D
stem and the anticodon stem form another continuous
double helix, also with a gap. Many of the invariant
and semi-invariant bases are involved in the tertiary
structure.

B. Complementary Sequence.

The complementary sequences will generally
consist of eleven nucleotides (see Figure 2) in two
blocks as shown, separated by two unpaired nucleotides
in the target sequence, preferably UU, wherein the two
blocks are complementary to a sequence 3' to the site
targeted for cleavage.

Ribozyme Activity

It is not necessary to provide ribozyme activity
if the cleavage is to occur intracellularly in the
nucleus since all cells contain RNAase P in their
nuclei. RNAase P must be supplied if cleavage is to
occur in the cytoplasm. As used herein for ease of
convenience, RNAase P refers to the ribonucleoprotein
consisting of the eukaryotic analogues of the *E. coli*
C5 protein and M1 RNA, regardless of source, whether
isolated, produced by chemical synthesis or, in the
case of the RNA, transcription from the gene, referred
to herein as H1 RNA. The RNA subunit need not
necessarily manifest catalytic activity in the absence
of protein subunits *in vitro*.

A. Endogenous RNAase P.

The sequence and proposed secondary structure of
H1 RNA, the RNA component of human RNAase P, was
reported by Baer, et al., in <u>Nucleic Acids Res.</u> 18(1),
97-103 (1989), the teachings of which are incorporated

herein.  The sequence of H1 DNA (**Sequence ID No. 1**) is
as follows:

ATGGGCGGAG GGAAGCTCAT CAGTGGGGCC ACGAGCTGAG TGCGTCCTGT
CACTCCACTCCCATGTCCCT TGGGAAGGTC TGAGACTAGG GCCAGAGGCG
GCCCTAACAG GGCTCTCCCTGAGCTTCAGG GAGGTGAGTT CCCAGAGAAC
GGGGCTCCGC GCGAGGTCAG ACTGGGCAGGAGATGCCGTG GACCCCGCCC
TTCGGGGAGG GGCCCGGCGG ATGCCTCCTT TGCCGGAGCTTGGAACAGAC
TCACGGCCAG CGAAGTGAGT TCAATGGCTG AGGTGAGGTA CCCCGCAGGG
GACCTCATAA CCCAATTCA<u>G ACCACTCTCC TCCGCCCATT</u>.

The nucleotide sequence of H1 DNA was determined
from the cDNA sequence.  The underlined part of the
sequence was determined by RNA sequence analysis.

Because of the similarity in secondary structure
and substrate specificity among the RNAase P's of
diverse origin, it is possible to use an EGS designed
to maximize efficiency of cleavage for the RNAase P in
question using techniques described herein to target
any RNA in any cell, even though the catalytically
active RNA subunits may have distinctly different
sequences.  See Altman, <u>Ann. Rev. Enzymology</u> 62, 1-
39(1989); Altman, <u>J. Biol. Chem.</u> 265, 20053-20056
(1990).  Secondary structure is defined by
intramolecular associations of complementary
sequences.  Base pairs can be canonical, A/U and G/C,
or non-canonical, G/U, A/G, etc.

**B.    Exogenous RNA having catalytic activity.**

An EGS can also be used in combination with an
RNA sequence that demonstrates enzymatic activity in
the presence or absence of a protein.  That RNA
sequence can be represented by a molecule like the
entire H1 RNA molecule or any portion thereof shown to
have catalytic activity in combination with a protein,
or any functionally equivalent molecule of eukaryotic
origin or derivation.

There are two principle situations in which
catalytic exogenous RNA or RNAase P is utilized in
combination with EGS: *in vitro* in the absence of

14

cells or cellular RNAase P and in circumstances
wherein the RNA to be cleaved is located in a portion
of a cell not containing endogenous RNAase P.  In the
latter case, the genes encoding the analogs of M1 RNA

5       and C5 protein (as defined above), or the human or
other eucaryotic equivalents thereof, are introduced
into the cell at the desired location for cleavage
using a suitable vector or other method known to those
skilled in the art for introduction and expression of

10      a gene in a cell.

**Application of the EGS as laboratory or clinical
reagents.**

The external guide sequences have applications
as *in vitro* reagents, in a similar fashion to

15      restriction enzymes, and as therapeutic agents, for
cleavage and inactivation of specific host cell RNA or
RNA coded for by pathogenic organisms such as bacteria
or viruses.  The external guide sequence, consisting
of the combination of eleven bases complementary to a

20      sequence on the 3' side of the desired cleavage site
and specific to the targeted RNA and a 3' terminal
NCCA sequence, can be added to any RNA having
sequences complementary to the EGS, in the presence of
RNAase P, and the RNA will be cleaved at the targeted

25      site.  In this manner, the activity of endogenous
RNAase P in any cell, such as the RNAase P of human
cells, can be directed to destroy specific messenger,
viral or other RNAs by the use of an appropriate EGS
RNA.

30      **1.   Reagents for *in vitro* applications.**

DNA restriction endonucleases are invaluable
reagents for the molecular biologist.  Patterns of
restriction fragment sizes are used to establish
sequence relationships between DNA molecules, and

35      large DNAs can be cleaved to give fragments of sizes
useful for genetic engineering, sequencing, and
studying protein binding.  RNA processing enzymes can

be utilized under conditions such that they also
cleave RNA with considerably sequence specificity.

Specific ribozymes can be prepared by combining
the specific guide sequence with eukaryotic RNAase P
or functional equivalents thereof.  In the preferred
embodiment, the external guide sequence and the RNA
subunit of RNAase P (e.g., H1 RNA) are separate;
alternatively, the two sequences can be combined using
an oligonucleotide linker that allows sufficient
flexibility between the targeting sequence and the H1
RNA (or equivalent) sequence for the targeting guide
sequence to bind and the catalytic sequence, whether
in association with an analog of C5 protein or not, to
cleave.

## 2.  Therapeutics.

### a.  Determination and Preparation of Complementary Sequences.

Any cellular gene product expressed as RNA,
including proteins encoded by mRNA and structural RNAs
themselves, can be targeted for inactivation by RNAase
P using sequences engineered to include appropriate
regions of sequence and/or structure for binding to
the targeted RNA and the desired site of cleavage.
The cellular gene product could be a modified product
of an oncogene, such as the ras gene product; where
the product is not a normal cell component, a viral
protein, such as one encoded by an essential gene for
HIV replication; or a bacterial protein.

In many cases, the critical genes in an
infective or pathological agent have been isolated and
sequenced.  Appropriate complementary sequences can be
synthesized using standard techniques, reagents, and
equipment based on these known sequences.

16

b.    Preparation of an appropriate
      pharmaceutical composition for delivery of
      the EGS to the targeted RNA.

There are two primary mechanisms for delivering
the EGS to intracellular RNA that has been targeted
for cleavage:   diffusion and via a vector.

As discussed above, any RNA that is important in
a disease process can be targeted and appropriate
complementary sequences made synthetically or by
copying cloned sequence.  Since RNAase P is
predominantly found in the nucleus of eukaryotic
cells, the infectious agents most likely to be
inhibited by administration of appropriate EGS to the
infected cells are those in which critical RNA
sequences are transcribed in the nucleus.  Important
examples of the viral agents that replicate in the
cell nucleus include herpesviruses (including herpes
simplex virus, varicella-herpes zoster virus,
cytomegalovirus, and Epstein-Barr virus), hepatitis B
virus, adenoviruses, paramyxoviruses such as measles,
and the retroviruses, such as human immunodeficiency
virus (HIV I, HIV II, HIV III and HTLV-1).
**Vector-mediated delivery of EGS.**

Preferred vectors are viral vectors such as the
retroviruses which introduce the EGS directly into the
nucleus where it is transcribed and released into the
nucleus.  Under the appropriate conditions, the EGS
will hybridize to the targeted RNA and the endogenous
RNAase P will cleave the hybridized RNA at the 5' side
of the hybrid region.

Methods for using retroviral vectors for gene
therapy are described in U.S. Patent Nos. 4,868,116
and 4,980,286, and PCT application PCT/US89/03794 and
PCT/US89/00422, the teachings of which are
incorporated herein.

Defective retroviral vectors, which incorporate
their own RNA sequence in the form of DNA into the

host chromosome, can be engineered to incorporate the EGS into the host, where copies will be made and released into the cytoplasm to interact with the target nucleotide sequences.

5    The ability to introduce specifically engineered nucleic acid sequences, as a means of targeted therapy, into hematopoietic cells of patients suffering from virus-induced disease of those cells, such as AIDS, has great potential. The most

10   efficacious methodology presently available for the introduction of specific genetic sequences into human cells involves the use of RNA-containing retroviruses which serve as vehicles or vectors for high efficiency gene transfer into human cells.

15   RNAase P-based therapy can also be used as a means of preventing the spread of HIV-1 and or providing a HIV-1 resistant population of T-cells that will be able to confer immune function to infected individuals. Patients who have been recently

20   diagnosed as having antibodies to HIV-1, but who do not yet show AIDS symptomatology, are the most likely be the best candidates for therapy. This procedure will necessitate removal of some of the patient's bone marrow stem cells and subsequent partial cytoblation.

25   The removed cells can be treated in the laboratory with appropriate EGS compositions (via appropriate viral vectors, such as defective viral vectors) and then restored to the same individual. The treated cells will develop in the patient into mature

30   hematopoietic cells, including T-cells. These T-cells will have normal immune function and, most importantly, will be intracellularly immunized to prevent their destruction by any HIV-1 still present in the patient.

35   Bone marrow stem cells and hematopoietic cells are relatively easily removed and replaced and provide a self-regenerating population of cells for the

propagation of transferred genes.  As described above,
HIV-1 and HTLV-1 should be amenable to these
approaches.  In the longer term, it is anticipated
that the use of RNAase P-based therapeutics will allow
the selective inactivation of other unwanted genes in
cells, such as activated oncogenes, involved in the
causation and maintenance of cancer cells.

In contrast to the approaches presently in use
which are aimed at preventing or limiting infection
with HIV, it should be possible to use RNAase P-based
technology to treat, and possibly to cure, HIV
infection, and related diseases of white blood cells
which are subject to transformation by retroviral
vectors carrying EGS.  Particular examples of diseases
that may be treated using EGS to target RNA for
cleavage by RNAase P include not only HTLV-1, but also
various retroviral-induced leukemias resulting from
chromosomal translocations that produce chimeric RNAs
which produce proteins that are unique to those cells
and that can act as growth stimulators or oncogenes.
Other types of transformed tissues that might be
treatable include all cancer cells carrying identified
oncogenes of known sequence.
Topical and other EGS compositions for direct
administration.

The EGS may also be administered topically or
systemically in a suitable pharmaceutical carrier.
Remington's Pharmaceutical Sciences, 15th Edition by
E.W. Martin (Mark Publishing Company, 1975), the
teachings of which are incorporated herein by
reference, discloses typical carriers and methods of
preparation.  The EGS may also be encapsulated in
suitable biocompatible microcapsules or liposomes for
targeting to phagocytic cells.  Such systems are well
known to those skilled in the art.

Therapeutically the oligoribonucleotides are
administered as a pharmaceutical composition

consisting of an effective amount of the EGS to
inhibit transcription of a targeted RNA and a
pharmaceutically acceptable carrier.  Examples of
typical pharmaceutical carriers, used alone or in
combination, include one or more solid, semi-solid, or
liquid diluents, fillers and formulation adjuvants
which are non-toxic, inert and pharmaceutically
acceptable.  Such pharmaceutical compositions are
preferable in dosage unit form, i.e., physically
discreet units containing a predetermined amount of
the drug corresponding to a fraction or multiple of
the dose which is calculated to produce the desired
therapeutic response, conventionally prepared as
tablets, lozenges, capsules, powders, aqueous or oily
suspensions, syrups, elixirs, and aqueous solutions.
It is essential that the oligonucleotides be delivered
in a form which prevents degradation of the
oligonucleotide before it reaches the intended target
site.  Examples of preferred means for delivery are
polymeric matrices such as the biodegradable polymers
polylactic acid and Pluronics.

A preferred composition is a topical
composition, for example, for application to a viral
lesion such as that produced by herpes simplex virus.
These will generally contain between 1 $\mu$M and 1 mM
oligonucleotide/unit of carrier, or produce a
concentration between 1 $\mu$M and 1 mM at the site of the
cells to be treated.  Oral compositions, although not
preferred, are in the form of tablets or capsules and
may contain conventional excipients such as binding
agents, (e.g., syrup, acacia, gelatin, sorbitol,
tragacanth or polyvinylpyrrolidone), fillers (e.g.,
lactose, sugar, corn starch, calcium phosphate,
sorbitol, or glycine), lubricants (e.g., magnesium
stearate, talc, polyethylene glycol or silica),
disintegrants (e.g., starch) and wetting agents (e.g.,
sodium lauryl sulfate).  Solutions or suspensions of

the EGS with conventional pharmaceutical vehicles are
employed for parenteral compositions, such as an
aqueous solution for intravenous injection or an oily
suspension for intramuscular injection.

5        For clinical applications, the dosage and the
dosage regimen in each case should be carefully
adjusted, utilizing sound professional judgment and
consideration of the age, weight and condition of the
recipient, the root of administration and the nature
10   and gravity of the illness.

The present invention, a RNA sequence targeting
a second RNA sequence for cleavage by eukaryotic
RNAase P, will be further understood by reference to
the following non-limiting examples.

15   **Example 1: Cleavage of tRNA precursor fragments by
human RNAase P in the presence of EGS.**

An EGS that can target RNA for cleavage by human
RNAase P was prepared from a small RNA fragment which
contains a 5' precursor sequence and the first
20   fourteen nucleotides from the 5' terminus of a tRNA
(Sequence ID No. 2, shown in Figure 3a). The leader
sequence of _E. coli_ tRNA$^{Tyr}$ precursor can be cleaved
correctly when another piece of RNA which contains the
remaining 3' proximal sequence of tRNA$^{Tyr}$ sequence is
25   hybridized to the target, as shown in Figure 3a and b,
demonstrating cleavage of a substrate by human RNAase
P in the presence or absence of EGS RNAs.

Human RNAase P was partially purified from HeLa
cells according to Bartkiewicz et al. (1989) Genes and
30   Development 3:488-499. The substrates were prepared
by _in vitro_ transcription in the presence of [$\alpha$-
$^{32}$P]GTP. [$\alpha$-$^{32}$P]GTP labelled pAva I RNA (28 nt) was
mixed with unlabelled EGS RNA and the mixture was
incubated at 37°C in 50 mM Tris-Cl (pH 7.5), 100 mM
35   NH$_4$Cl, and 10 mM MgCl$_2$ with enzyme for 30 min.
Labelled pAva I RNA alone was also incubated with or
without enzyme. Analysis by gel electrophoresis shows

that the EGS plus enzyme resulted in cleavage of the
RNA.

The 3' fragment is the external guide sequence
(EGS). Because the lengths of the leaders and their
sequences, as well as the sequences of the mature
domain, are not conserved among different precursor-
tRNAs, the main determinants for human RNAase P
cleavage must be in some of the conserved structural
features of various tRNAs. This general idea is borne
out by the fact that several other EGSs that did not
mimic exactly the structure of parts of a tRNA (i.e.,
changes in the number of possible base pairs in the D
or amino acyl stems, changes in positions 8 and 9 of
the mature tRNA sequence, and a change from cytosine
to uracil at position 57) did not target complementary
RNAs. However, an EGS that lacked the anticodon stem
and loop, or the variable stem and loop, resulted in
efficient cleavage, indicating that these parts of the
EGS, separately, were not essential for recognition of
the target complex by the enzyme.

Presumably, if an mRNA, rather than part of a
precursor tRNA sequence, is incorporated into the
double-stranded stem region of a putative target
complex, and the resulting hybrid contains the
structural features required of a substrate for human
RNAase P activity, the mRNA should be cleaved by human
RNAase P.

**Example 2: Specific cleavage of CAT mRNA *in vitro* by
human RNAase P using an external guide
sequence.**

The mRNA for the gene for chloramphenicol
acetyltransferase (CAT) can be easily manipulated on
plasmids and the enzymatic activity is readily
expressed in tissue culture cells so it was used as a
target substrate. As demonstrated below, an EGS can
target CAT mRNA for specific cleavage by human RNAase
P. Figure 4 shows a complex in which an EGS (Sequence

ID No. 3) could base-pair with nucleotides 67 to 79 of
CAT mRNA and direct human RNAase P to cleave that mRNA
at nucleotide 67 (The first nucleotide of translation
initiation codon is numbered 1). The EGS$^{CAT}$ construct

5    was derived from the _E. coli_ tRNA$^{Tyr}$ gene where the
first eighteen nucleotides from the 5' terminus have
been deleted and the sequences on the D-loop and
acceptor-loop have been changed to make base-pairs
with CAT mRNA. The EGS$^{CAT}$ fused upstream with a T7

10   promoter was cloned into a pUC19 vector. The EGS$^{CAT}$
RNA was prepared through _in vitro_ transcription with
T7 RNA polymerase. A _Hind_ III - _Bam_ HI DNA fragment
of the CAT gene (pCAT$^{TM}$, Promega) was cloned in pGem-2.
The CAT DNA was linearized with _EcoRI_ and transcribed

15   with T7 RNA polymerase in the presence of [$^{32}$P-$\alpha$]GTP.
Labeled CAT mRNA was mixed with various amounts of the
EGS$^{CAT}$ molecule without special treatment of denaturing
or annealing and susceptibility to cleavage by RNAase
P _in vitro_ was tested with human RNAase P partially

20   purified from HeLa cells.

The _Hind_ III -- _Bam_ HI fragment of CAT gene
(pCAT$^{TM}$, Promega) was cloned in pGem-2. The plasmid
was truncated with _EcoRI_ and a 260 nucleotide-long
transcript was obtained by _in vitro_ transcription with

25   T7 RNA polymerase in the presence of [$\alpha$-$^{32}$P]GTP. The
EGS sequence was synthesized by polymerase chain
reaction, using the _E. coli_ tRNA$^{Tyr}$ gene as template,
with oligonucleotide 5'-GCCAAACTGAGCAGACTC-3' and 5'-
GCGCggtacc__AAAAA__TGGTGAGGCATGAAGG-3', where the bold

30   letters in the oligonucleotide sequences indicate the
bases needed to make base pairs to CAT mRNA, the
underlined letters indicate the sequence complementary
to the transcription termination signal, and the lower
case letters shows an extra linker sequence (GCGC at

35   the 5' end of the second oligonucleotide are extra
nucleotides). The PCR fragment was digested with _Hind_
III and cloned into pUC19 with a T7 promoter upstream

from the EGS sequence. The EGS<sup>CAT</sup> RNA was transcribed
with T7 RNA polymerase after the plasmid was
linearized with *Dra* I. A mixture of unlabelled and
[$\alpha$-$^{32}$P]GTP labelled CAT mRNA fragment (0.2 pmole in
total) was mixed with the EGS<sup>CAT</sup> RNA in amount of 4
pmole, 1 pmole, 1 pmole, 0.4 pmole and 0.2 pmole.
Each mixture was incubated at 37°C in 50 mM Tris-Cl
(pH 7.5), 100 mM NH$_4$Cl, and 25 mM MgCl$_2$ with enzyme for
one hour. The reaction was stopped by addition of an
equal volume of dye solution with excess EDTA and then
subjected to a 5% polyacrylamide-7M urea gel. CAT
mRNA alone was incubated without and with enzyme and
loaded on the gel.

Primer extension analysis determining the
precise site of EGS<sup>CAT</sup>-directed cleavage by human
RNAase P was conducted as follows. A reverse
transcription reaction was performed on the uncleaved
and cleaved CAT mRNA using an oligodeoxyribonucleotide
5'GGCCGTAATATCCAGCTGAACGG 3', complementary to
nucleotide 129-107 of CAT mRNA. The reaction was
incubated in 100 mM Tris.HCl (pH 8.3), 10 mM KCl, 6 mM
MgCl$_2$, 10 mM DTT and 2 units AMV reverse transcriptase
at 46°C for 2 hours. Labelled G, A, U, C were used as
reference analyses of DNA sequences corresponding to
the CAT mRNA template.

The precise site of cleavage of CAT mRNA was
determined by primer extension analysis using an
oligodeoxyribonucleotide primer complementary to
nucleotide 129-107 of the RNA shows that the cleavage
occurs between nucleotides 66 and 67, as expected.

The results of the EGS<sup>CAT</sup> RNA directed cleavage
of chloramphenicol acetyltransferase (CAT) mRNA were
analyzed by gel electrophoresis. In the presence of
EGS<sup>CAT</sup> molecules, CAT mRNAs were cleaved to give rise
to two products with the expected size. Analysis of
the products of the reaction showed that the end
groups contained 5' phosphoryl and 3' hydroxyl

24

termini, the same as those normally generated by
RNAase P.  The results show conclusively that the
specific cleavage of CAT mRNA is due to an EGS-
directed RNAase P hydrolytic reaction.

For up to five-fold molar excess of EGS$^{CAT}$ RNA to
mRNA, the cleavage efficiency is proportional to the
amount of EGS$^{CAT}$ added.  However, more than ten-fold
excess of EGS$^{CAT}$ molecules caused a decrease in the
cleavage efficiency.  One explanation for this is that
EGS$^{CAT}$ alone inhibits the enzymatic activity by
competing with the mRNA-EGS complex for the enzyme.
The reaction proceeds in a linear fashion for more
than 3 hours at 37°C.  Denaturation and reannealing of
the oligonucleotides in the target complex did not
improve the efficiency of cleavage.  The reaction has
an absolute requirement for Mg$^{2+}$ with an optimal
concentration of 25 mM, in contrast to that with tRNA$^{Tyr}$
precursor as substrate, which has an optimal Mg$^{2+}$
concentration of 2-10 mM.

**Example 3: Inhibition of Expression of CAT activity in
green monkey CV-1 cells by EGS$^{CAT}$.**

In order to test whether the EGS can function *in
vivo*, the EGS$^{CAT}$ sequence was inserted downstream of a
mouse U6 snRNA gene promoter in a Bluescript™ vector.
The EGS$^{CAT}$ sequence can be transcribed by RNA
polymerase III and the transcription can terminate at
a T$_5$ cluster following the EGS sequence in either S100
extract or living cells.  Green monkey fibroblast
cells CV-1 were cotransfected with pCAT pEGS$^{CAT}$
plasmids.  After transfection with pCAT and pEGS$^{CAT}$,
cells were harvested and CAT activity was assayed.

CV-1 cells were maintained in Dulbecco's
modified Eagle medium that contained 10% fetal calf
serum.  One day prior to transfection cells were split
1:10 and plated in 60 mm Petri plates.  Two hours
prior to transfection cells were fed with 4.5 ml of
fresh medium with 10% fetal calf serum.  Transfection

was performed using the calcium phosphate
precipitation procedure using 2.5 µg of pCAT DNA and
various amounts of pEGS<sup>CAT</sup> DNA, ranging from one to
6.25 µg. Twenty-four hours after transfection, cells
were harvested and cell extracts were assay for CAT
activity.

The extract from cells cotransfected with EGS<sup>CAT</sup>
construct apparently decreased the conversion of
chloramphenicol to its acetylated forms. The degree
of inhibition was measured quantitatively by counting
of the spots excised from a TLC plate. EGS<sup>CAT</sup>
cotransfection produced greater than 50% inhibition
compared to the control with no EGS<sup>CAT</sup> cotransfection.
There was substantial loss of ability to inhibit CAT
expression when a higher ratio of pEGS<sup>CAT</sup> to pCAT was
introduced. Similar experiments yielding
approximately 70% of CAT activity have also been
performed with human cells in tissue culture.

**Example 4: Preparation of Modified EGS enhancing
degradation of target RNA by RNAase P.**

Two classes of EGS were designed, as shown in
Figures 5 and 6. The first class involves deletions
of large segments of the EGS as suggested in Example 1
and described in Examples 2 and 3. It has been found
that the anticodon loop and stem can be deleted, as
shown in Figure 5b (Sequence ID No. 4) and the EGS
still promote cleavage of a target RNA by RNAase P.
The variable loop and part of the variable loop can
alternatively be deleted from the EGS and the EGS will
promote cleavage with greater efficiency than the
parent EGS molecule. The most efficient EGS was the
one in which the anticodon stem and loop was deleted.
This EGS promoted cleavage by human RNAase P of a
target mRNA (CAT mRNA) at a rate 10-fold higher than
the parent EGS. These deletions cannot be combined,
however, to make an efficient EGS.

The second class of EGSs have changes in both
the equivalent of the T loop, the variable loop, and
the anticodon stem of the tRNA-like segment of the
EGS. Three EGSs are shown in Figures 6a (Sequence ID
No. 5), 6b (Sequence ID No. 6), and 6c (Sequence ID
No. 7), respectively. Figure 6c shows the most
efficient EGS, which directs cleavage by human RNAase
P of a target RNA (CAT mRNA) in the appropriate
complex at a rate approximately fifty to one hundred
fold greater than the parent EGS.

These results apply to relative rates of
cleavage at a particular site in a target mRNA. The
absolute rates of cleavage at any particular site
still depends on access of the EGS to that particular site.

**Example 5: Preparation of RNAs with randomized**
**nucleotides.**

Double stranded DNA templates were made by
annealing of and enzymatically extending two
overlapping synthetic oligonucleotides:
TAATACGACTCACTATAGAACATTTTGAGGCATTTCAGTCAGTTGGCCAAACTG
AGCAGAC (Sequence ID No. 8) and
TGGTGAGGCATGAAGGNNNNGAACCTTCNNNNNGCAGATTTAGAGTCTGCTCAG
TTTGGCC (Sequence ID No. 9), where the complementary
sequences are underlined and the randomized
nucleotides N were introduced during their machine
synthesis by incorporating equimolar quantities of
four nucleotides. These sequences create a chimeric
tRNA gene that contains sequences from CAT mRNA and
tRNA$^{Tyr}$ from *E.coli*. A promoter for T7 bacteriophage
RNA polymerase is included in Sequence ID No. 8. The
extension was carried out with AMV reverse
transcriptase at 46°C for two hours. Variant RNA
pools were prepared by transcription with T7
polymerase in 40 mM Tris.Cl, pH 7.9, 6 mM MgCl$_2$ 10 mM
dithiothreitol 2 mM spermidine 1 mM NTPs containing 20
μCi [$^{32}$-α] GTP at 37°C.

**The selection procedure.** RNA substrates were subjected to digestion with 30 units (one unit is defined as the amount of human RNAase P that will digest 1 pmol of tRNA precursor in 30 min at 37°C) for 2 hrs in the first three rounds of the selection procedure. For subsequent rounds of selection, the amount of the enzyme was reduced and incubation time was shortened so that less than 20% of substrate was cleaved. Cleaved products were separated from uncleaved substrates by electrophoresis on a 8% polyacrylamide/7 M urea gel. The purified product RNAs were reverse transcribed and amplified by PCR with Sequence ID No. 8 and TGGTGAGGCATGAAGG (Sequence ID No. 10) as primers using the Perkin Elmer RNA PCR kit. After each round of selective amplification, random mutations were introduced by performing the PCR with an error rate of 0.1% per each nucleotide position. PCR-generated double stranded DNAs regained the T7 promoter and leading sequence from primer Sequence ID No. 8, and were used to transcribe RNA for the next round of selection.

**Characterization of the selected RNAs and EGS RNA derived from them.** After eight cycles of selection, the resulting double-stranded DNAs were cloned into the Bluescript vector (Promega). Eighteen plasmid DNAs were sequenced using Sequenase 2.0 (U.S. Biochemicals, Cleveland, OH).

In order to test the abilities of EGSs derived from the individual variants selected above for CAT mRNA targeting, sequences corresponding to the EGS segment of each chimeric tRNA were amplified by PCR using primers Sequence ID No. 8 and AATACGACTCACTATAGGCCAACTGAGCAGAC (Sequence ID No. 11), which contains a promoter sequence for T7 polymerase, and RNAs were transcribed with T7 RNA polymerase. EGS-directed CAT mRNA cleavage was assayed in 10 $\mu$l of 50 mM Tris.Cl, pH 7.5, 10 mM $MgCl_2$ 100 mM $NH_4Cl$

28

containing 0.25 pmol (1000 cpm) of substrate RNA and 1
or 5 pmol of EGS RNAs. Reaction mixtures were
incubated at 37°C for 30 min with 10 units of RNase P
from HeLa cells, followed by electrophoresis in 5%
polyacrylamide/7 M urea gels.

The gels show a species of RNA migrating in the
position expected for cleavage of substrate RNA in
those lanes where the newly selected EGSs have been
included in the reaction mixtures.

This method can be utilized to produce efficient
EGSs for any RNAase P by randomizing other parts of
the parent EGS sequence to select for EGSs with
sequences other than those described herein that may
be even more efficient.

**Summary of data in examples.**

Although messenger RNAs are not natural
substrates of RNAase P, complexes of mRNA-EGS have a
common feature with precursors to tRNA in secondary or
tertiary structure that forms the basis for the
specificity of RNA cleavage by RNAase P. Given the
secondary structures associated with RNA molecules,
the choice of target site may play a crucial role in
determining whether the substrate is accessible to EGS
binding. Some target regions may participate in a
high order structure which prevents the mRNA from
binding EGS. Since high order structures can hinder
the accessibility of certain sequences in a potential
target to an EGS, some general methods, such as
sensitivity to nucleases specific for single-stranded
regions and analysis of sequences for regions required
to be accessible to components of the translation
apparatus, may be necessary to scan for suitable
target sites in mRNA.

The method established in this work enables one
to direct human RNAase P to cleave any target RNA *in
vivo* with an appropriately designed proper external
guide sequence (EGS). The method may provide a

powerful new tool to block the function of specific
RNAs. Theoretically, specific degradation of certain
RNAs by RNAase P should be more powerful than the
antisense RNA technique because RNAase P destroys the
5     targeted RNA irreversibly and RNAase P, as well as the
EGS, act catalytically since they can recycle many
times, Li, et al., Proc. Natl. Acad. Sci. USA 89,
3185-3189 (1992) and Perreault and Altman, J. Mol.
Biol. 226, 399-409 (1992).

10    Modifications and variations of the method and
compositions to target any RNA for cleavage by
eukaryotic RNAase P will be obvious to those skilled
in the art from the foregoing detailed description.
Such modifications and variations are intended to come
15    within the scope of the appended claims.

We claim:

1. A composition for targeting an RNA substrate for cleavage by eukaryotic RNAase P comprising a recombinant external guide sequence molecule including

a cleavage targeting sequence, and

a nucleotide sequence complementary to the substrate,

wherein the external guide sequence base pairs to the substrate to form a hybrid structure having secondary structure resembling a precursor tRNA under conditions promoting cleavage by the RNAase P of the substrate at the nucleotide at the 5' end of the base-paired region.

2. The composition of claim 1 wherein the cleavage targeting sequence comprises a D loop and stem, T stem and loop, aminoacyl stem, and either an anticodon stem and loop or variable loop.

3. The composition of claim 1 wherein the complementary sequence is at least eleven nucleotides in length and comprises seven nucleotides base pairing with the targeted sequence, followed by two nucleotides not base pairing with the targeted sequence, followed by four nucleotides base pairing with the targeted sequence.

4. The composition of claim 1 wherein the RNAase P is selected from the group consisting of yeast and mammalian RNAase P.

5. The composition of claim 1 wherein the D stem and aminoacyl stem of the external guide sequence base pair with sequence on the targeted RNA and are separated by two unpaired nucleotides.

6. The composition of claim 2 wherein the external guide sequence comprises an anticodon stem and loop or portions thereof.

7.   The composition of claim 2 wherein the external guide sequence comprises a variable stem and loop or portions thereof.

8.   The composition of claim 1 wherein the external guide sequence is modified in a region selected from the group consisting of the T loop., anticodon stem and loop or variable stem and loop.

9.   The composition of claim 1 wherein the RNAase P is targeted to sequences such that cleavage of the sequences by the RNAase P results in inactivation of RNA selected from the group consisting of oncogenes, tumor suppressor genes, viral genes, and cellular mRNAs which encode proteins selected from the group consisting of enzymes, hormones, cofactors, antibodies, and growth factors.

10.   The composition of claim 1 further comprising a pharmaceutical carrier selected from the group consisting of carriers suitable for topical, subcutaneous, parenteral, and enteral administration.

11.   The composition of claim 1 further comprising a vector for introducing the external guide sequence into a cell containing the RNA targeted for cleavage.

12.   The composition of claim 11 wherein the vector is a retroviral vector.

13.   A method for specifically cleaving RNA comprising providing in combination with RNAase P, an external guide sequence including

a cleavage targeting sequence, and

a nucleotide sequence complementary to the substrate,

wherein the external guide sequence base pairs to the substrate to form a hybrid structure having secondary structure resembling a precursor tRNA under conditions promoting cleavage by the RNAase P of the substrate at the nucleotide at the 5' end of the base-paired region.

14. The method of claim 13 wherein the cleavage targeting sequence comprises a D loop and stem, T stem and loop, aminoacyl stem, and either an anticodon stem and loop or variable loop.

15. The method of claim 13 wherein the complementary sequence is at least eleven nucleotides in length and comprises seven nucleotides base pairing with the targeted sequence, followed by two nucleotides not base pairing with the targeted sequence, followed by four nucleotides base pairing with the targeted sequence.

16. The method of claim 14 wherein the external guide sequence comprises an anticodon stem and loop or portions thereof.

17. The method of claim 14 wherein the external guide sequence comprises a variable stem and loop or portions thereof.

18. The method of claim 14 wherein the external guide sequence is modified in the T loop, anticodon stem and loop, or variable stem and loop.

19. The method of claim 13 wherein the RNAase P is selected from the group consisting of yeast and mammalian cell RNAase P.

20. The method of claim 13 wherein the targeted RNA is intracellular and the RNAase P is endogenous to the cell.

21. The method of claim 13 further comprising providing an RNAase P functional equivalent with the external guide sequence.

22. The method of claim 13 wherein the RNAase P is targeted to sequences such that cleavage of the sequences by the RNAase P results in inactivation of RNA selected from the group consisting of oncogenes, tumor suppressor genes, viral genes, and cellular mRNAs which encode proteins selected from the group consisting of enzymes, hormones, cofactors, antibodies, and growth factors.

23. The method of claim 22 further comprising providing the external guide sequence in combination with a pharmaceutical carrier selected from the group consisting of carriers suitable for topical, subcutaneous, parenteral, and enteral administration.

24. The method of claim 13 further comprising providing the external guide sequence in combination with a vector for introducing the external guide sequence into a cell containing the RNA targeted for cleavage.

25. A method for selecting a population of external guide sequences for cleavage of a target RNA by RNAase P with increased efficiency over a starting external guide sequence comprising

randomizing a section of the starting external guide sequence;

selecting for a subpopulation of the randomized sequences for their ability to be cleaved efficiently by RNAase P;

amplifying those sequences cleaving more efficiently than the starting external guide sequence; and

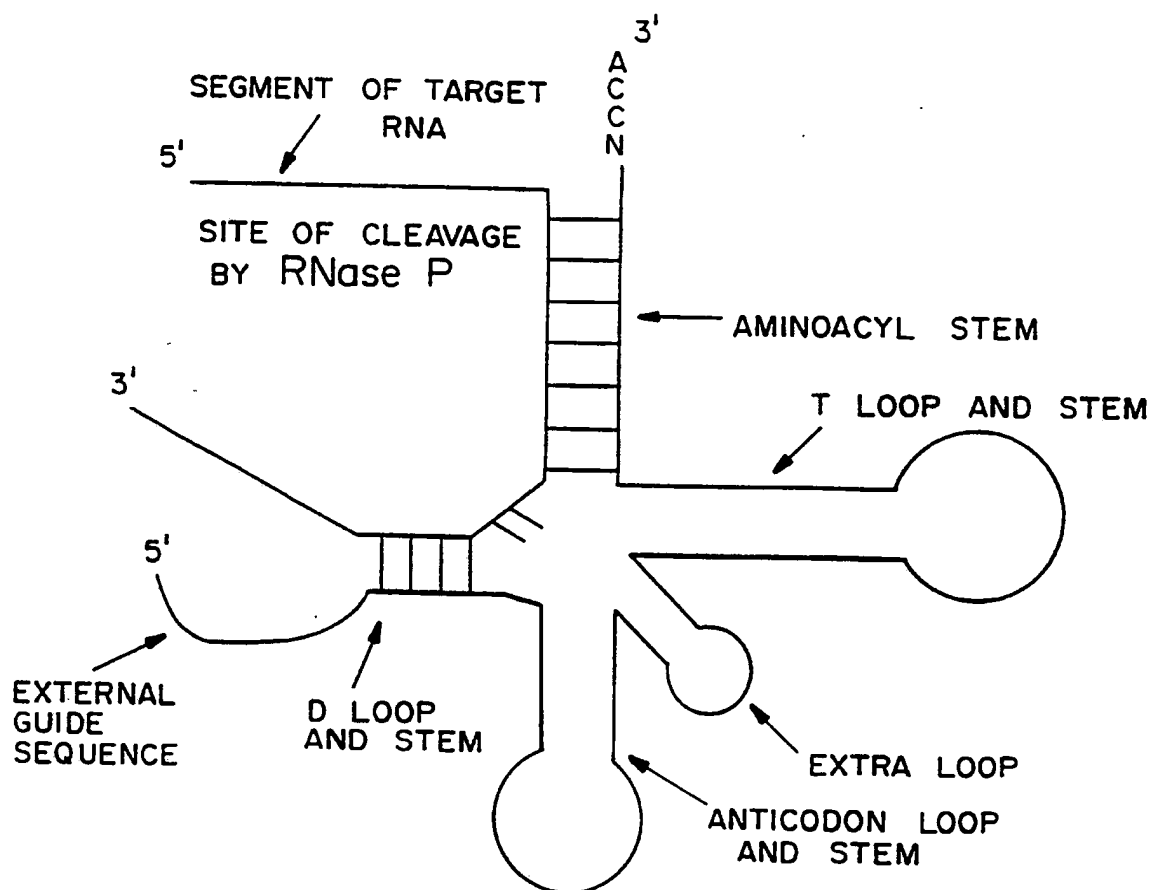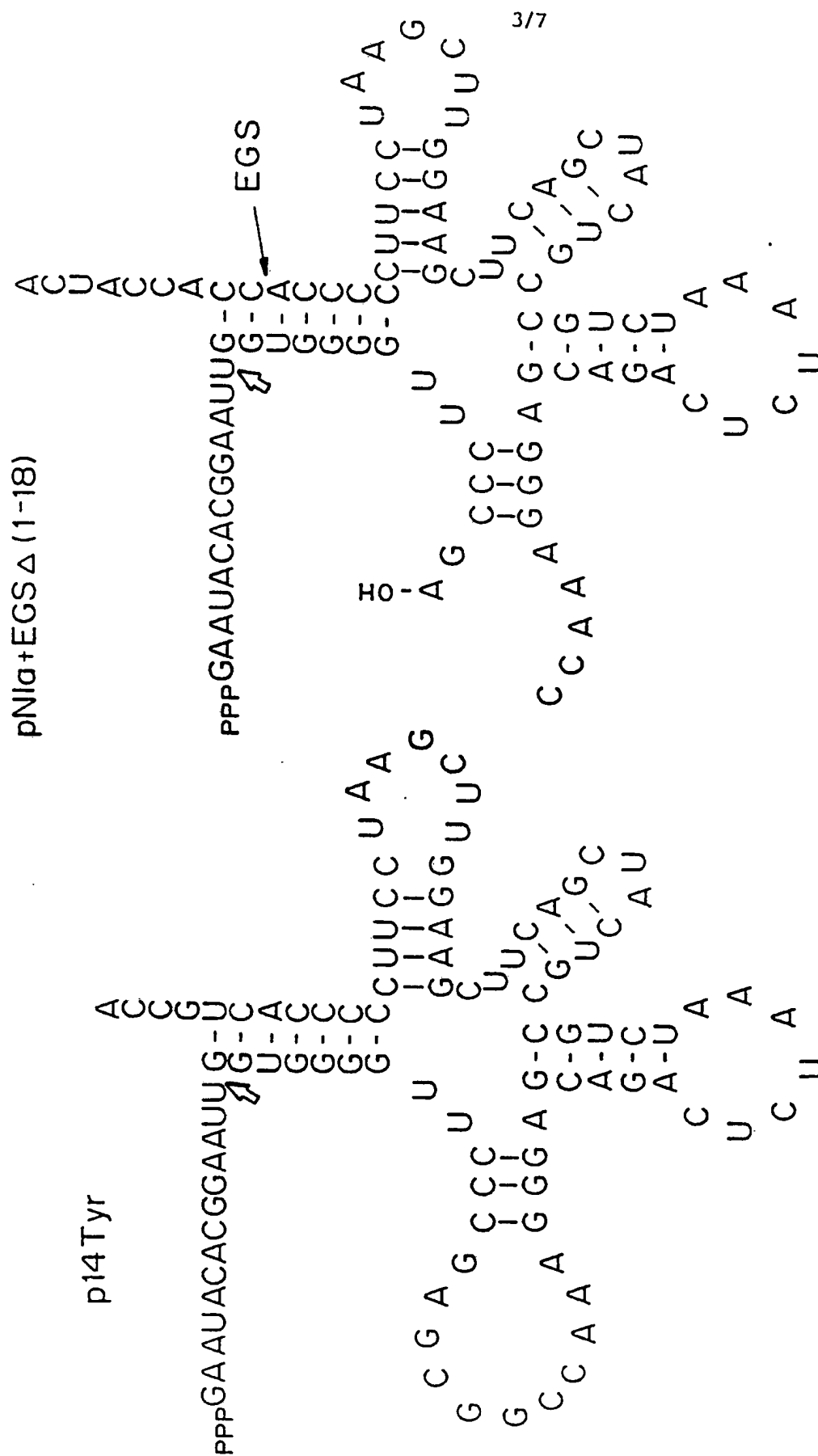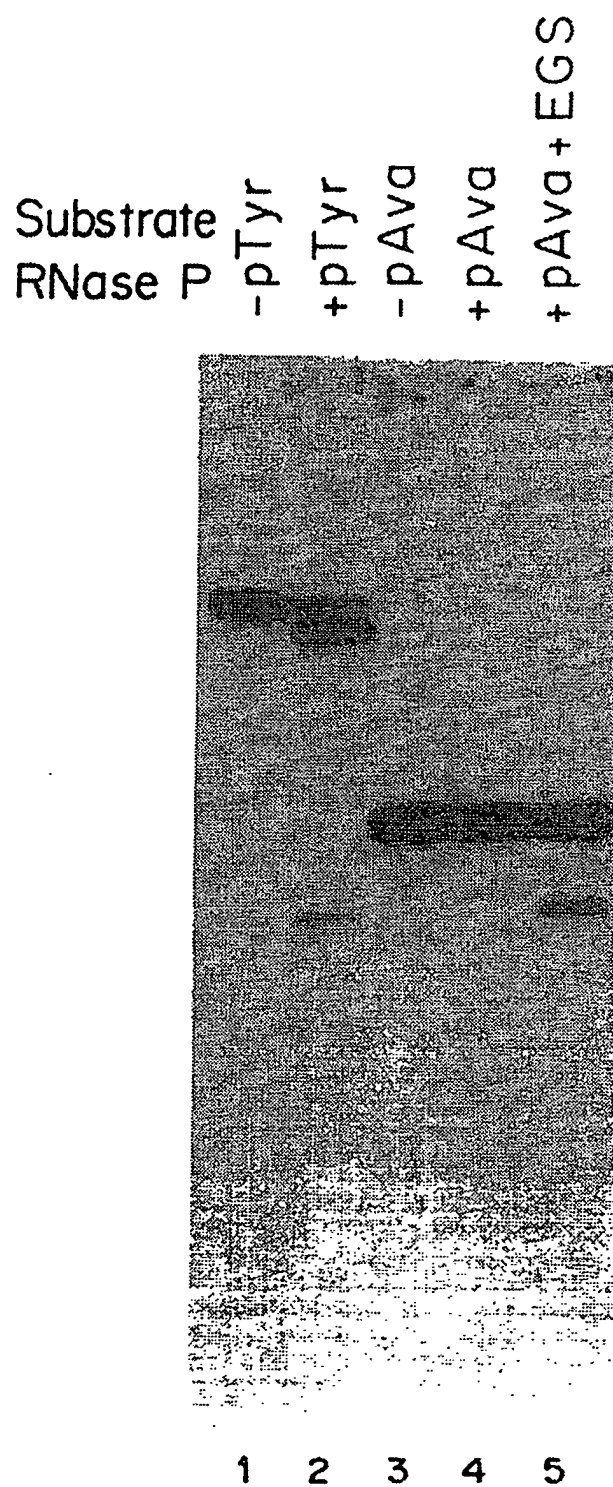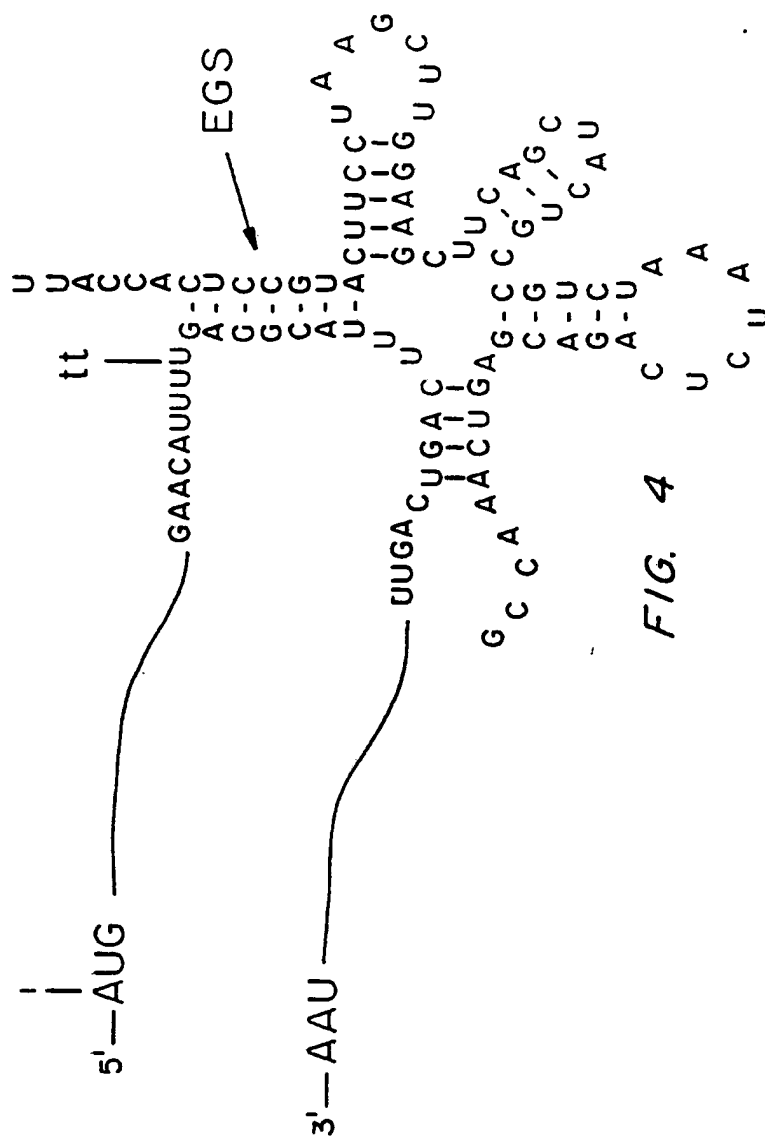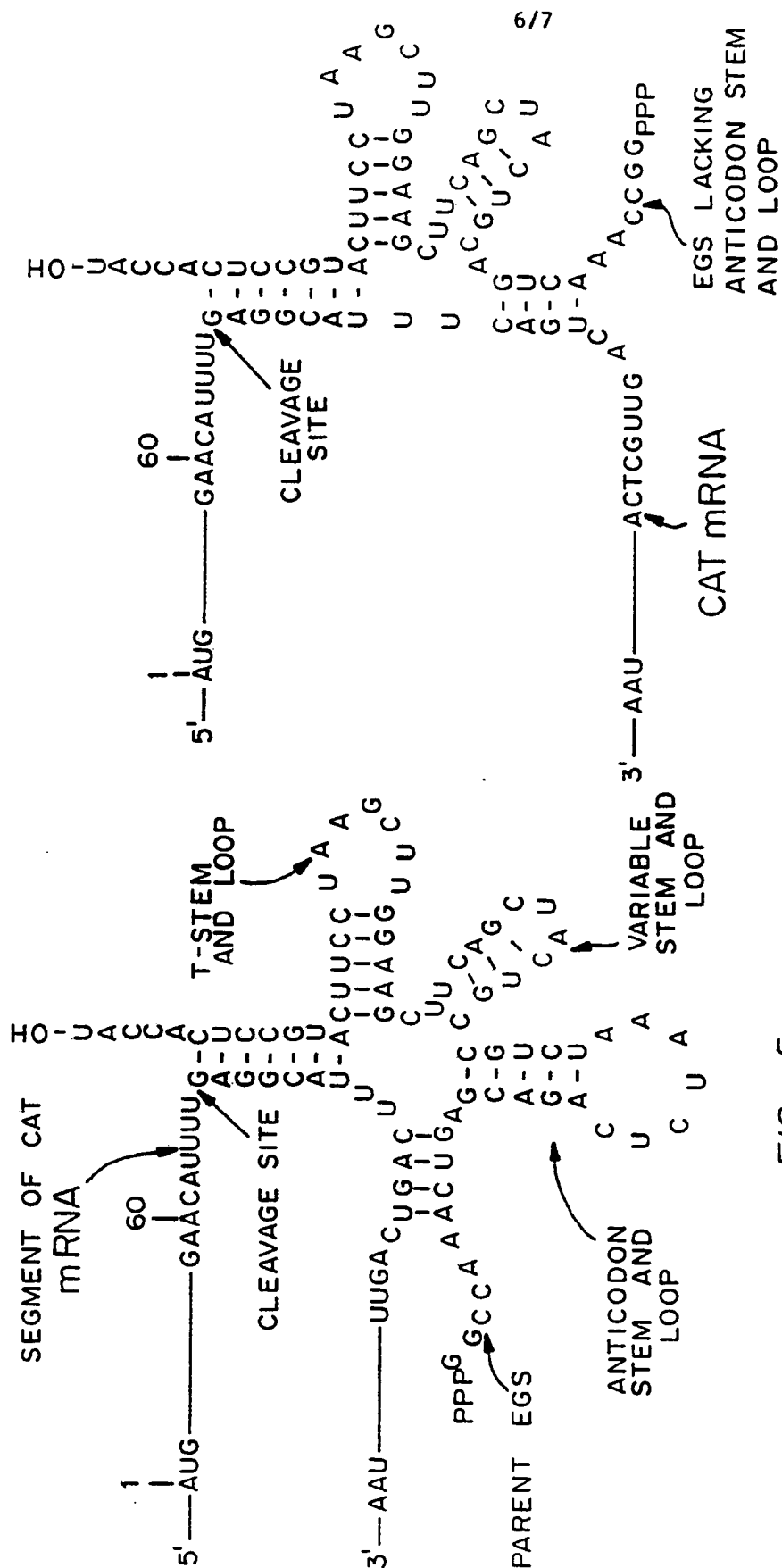repeating the selection and amplification steps.
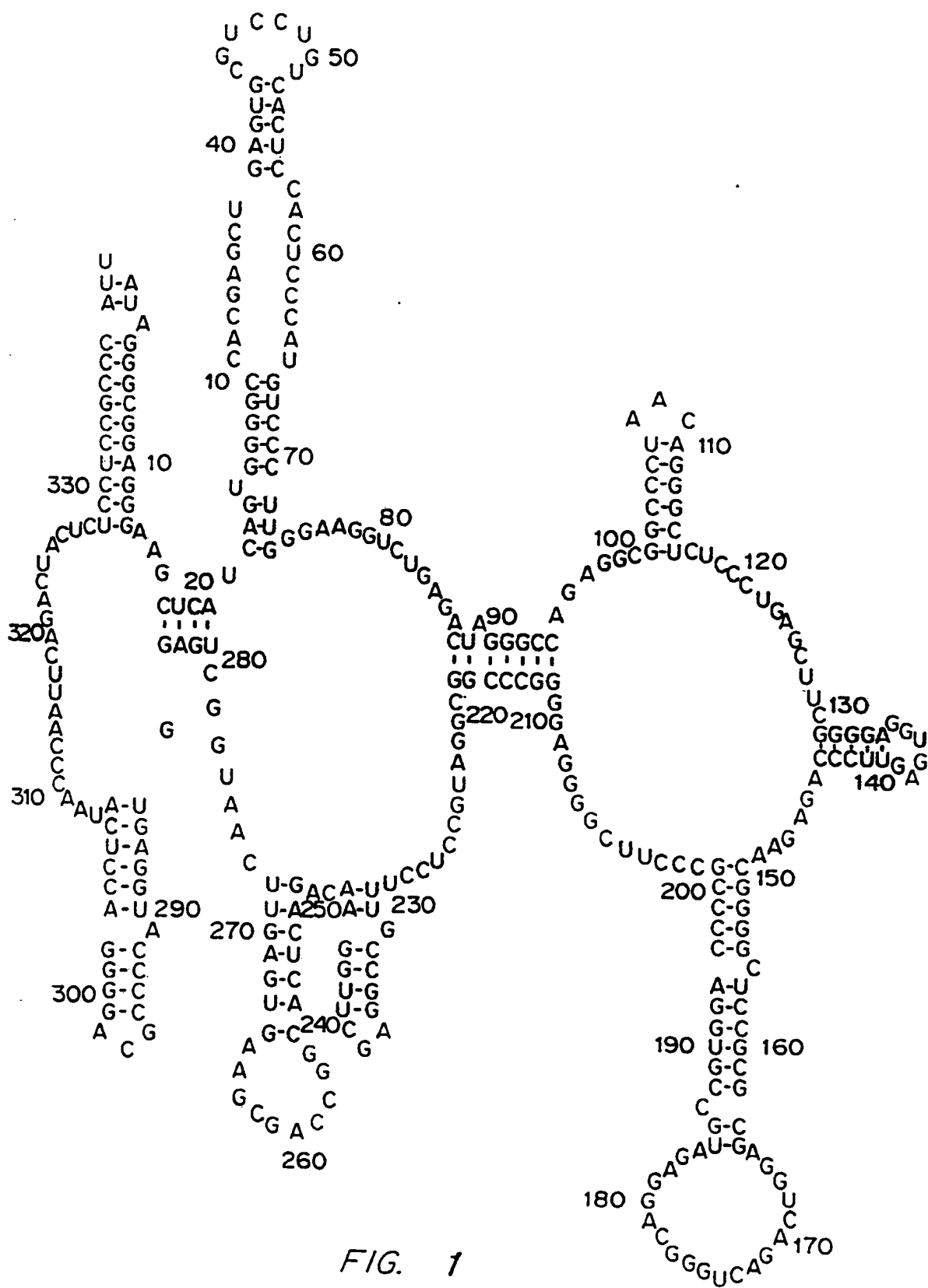
FIG. 1

FIG. 2

FIG. 3a

FIG. 3b

FIG. 4

FIG. 5a

FIG. 5b

FIG. 6a

FIG. 6b

FIG. 6c